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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Bacterial Surface Protein Expression

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Notice: This application is as filed and may therefore contain an incomplete specification.

Canada

Abstract

This invention provides a bacterium having an S-lay r wherein the bacterium's S-layer prot in gene contains n or more sequences encoding one or more heterologous polypeptides and, said S-layer is a fusion product of the S-layer protein and said heterologous polypeptide. Preferably, the bacterium is a Caulobacter which may be cultured as a film in a bioreactor or may be administered as a live vaccine. The heterologous polypeptide may be an enzyme, a ligand, an antigen or another functional sequence of amino acids. This invention also provides a method of expressing and presenting a functional polypeptide which comprises cloning a gene sequence for said polypeptide in-frame into the S-layer protein gene of Caulobacter and, culturing said Caulobacter having an S-layer which is a fusion product of the S-layer protein and the polypeptide. This invention also provides a DNA sequence comprising all or substantially all of a Caulobacter S-layer protein gene containing one or more heterologous sequences encoding restriction endonuclease sites which heterologous sequences preserve the translational frame of said gene to facilitate insertion of further heterologous sequences into said gene. This invention also provides a substantially purified DNA molecule essentially consisting of all or substantially all of a Caulobacter crescentus S-protein gene sequence.

The embodiments of the invention in which an xclusive property or privilege is claimed are defined as foll ws:

1. A bacterium having an S-layer wherein the bacterium's S-layer protein gene contains one or more sequences encoding one or more functional heterologous polypeptides and, said S-layer is a fusion product of the S-layer protein and the said heterologous polypeptides.

2. The bacterium of claim 1 wherein the bacteria is a Caulobacter.

3. The bacterium of claim 1 wherein the bacterium is C. crescentus.

4. The bacterium of claim 1, 2, or 3 wherein the bacterium is a live vaccine.

5. The bacterium of claim 1, 2, or 3 wherein the heterologous polypeptide is an enzyme.

6. The bacterium of claim 5 wherein the enzyme is capable of degrading wood.

7. The bacterium of claim 6 wherein the enzyme is cellulase or xylanase.

8. The bacterium of claim 1, 2, or 3 wherein the heterologous polypeptide is a metal binding ligand.

9. The bacterium of claim 8 wherein the heterologous polypeptide is a metallothionein.

10. A bioreactor comprising a substrate and, a multiplicity of the bacterium of claim 1, 2, 3, 6, 7, or 9 adhered thereon.

11. The bioreactor of claim 10 wherein th heterologous polypeptide is an enzyme.

12. The bior actor of claim 10 wh r in the h t r log us polypeptide is a metal binding ligand.

13. A method of expressing and presenting a functional polypeptide which comprises cloning a gene sequence for said polypeptide in-frame into the S-layer protein gene of Caulobacter, and, culturing said Caulobacter having a S-layer which is a fusion product of the S-layer protein and the polypeptide.

14. The method of claim 13 wherein said Caulobacter is cultured as a film in a bioreactor.

15. The method of claim 13 wherein the functional polypeptide is selected from the group consisting of a metailothionein, cellulase and xylanase.

16. A fusion product comprising a S-layer protein and one or more functional polypeptide sequences expressed according to the method of claim 13 or 14 wherein the fusion product is separated from the Caulobacter cell surface after expression thereof.

17. Caulobacter rsaA gene that expresses S-layer protein, wherein the expressed part of the gene contains one or more heterologous restriction endonuclease sites that preserve the translational frame of the gene.

18. A plasmid containing the gene of claim 17.

19. A bacterium containing the gene of claim 17.

Bacterial Surface Protein Expression

This invention relates to the expression of heterologous proteins, or portions of such proteins, from cloned genes in bacteria wherein the expressed protein is fused with a surface protein of the bacterium.

Bacterial surface proteins have been used as carriers or vehicles of foreign epitopes expressed in the bacterium (particularly in Salmonella and E. coli) for various purposes, including the development of live vaccines. In some instances, the heterologous protein is expressed as a fusion product with a surface protein of the bacterium. Generally, the use of such surface proteins as a vehicle for expression and presentation of heterologous proteins has been limited by the characteristics of the particular surface protein involved. The bacterium's lipopolysaccharide layer, which tends to stimulate a strong immune response, also covers the integral outer membrane proteins of the organism and potentially affects efficient presentation of a cloned epitope. Also, where the surface protein is functional, for example, as part of a filamentous portion of the bacterial cell surface, there will be limited opportunities to express a fusion product and still retain the surface protein's function. Generally, the organisms that have been used for these purposes have been chosen because of the advantages presented in respect of the organism's relationship to its host.

Many genera of bacteria assemble layers composed of repetitive, regularly aligned, proteinaceous sub-units on the outer surface of the cell. These layers are essentially two-dimensional paracrystalline arrays, and being the outer molecular layer of the organism, directly interface with the environment. Such layers are commonly known as S-lay rs and are found on memb rs of every

taxonomic group of walled bacteria including: Archaebacteria; Chlamydia; Cyanobacteria; Acin tobacter; Bacillus; Aquaspirrillum; Caulobacter; Clostridium; Chromatium. (see: Smit, J.; PROTEIN SURFACE LAYERS OF BACTERIA; in:"Offprints From Bacterial Outer Membranes As Model Systems" (1986) Dr. M. Inouye (Ed.); John Wylie and Sons, Inc.).

Typically, an S-layer will be composed of an intricate, geometric array of at least one major protein having a repetitive regular structure. In many cases, such as in Caulobacter, the S-layer protein is synthesized by the cell in large quantities and the S-layer completely envelopes the cell and thus appears to be a protective layer.

Caulobacter bacteria are natural inhabitants of most soil and freshwater environments and may persist in waste water treatment systems and effluents. The bacteria alternate between a stalked cell that is attached to a surface and a motile dispersal cell that has adhesive material already expressed and is searching to find a new surface upon which to stick and convert to a stalked cell: The bacteria attach tenaciously to nearly all surfaces and do so without producing the extracelluar enzymes or polysaccharide "slimes" that are characteristic of most other surface attached bacteria. They have simple requirements for growth. The organism is ubiquitous in the environment and has been isolated from oligotrophic to mesotrophic situations. Caulobacters are known for their ability to tolerate low nutrient level stresses, for example, low phosphate levels. This nutrient can be limiting in many leachate waste streams, especially those with high levels of iron or calcium.

The S-layer of Caulobacter crescentus has been well characterised. N arly all freshwater isolates of

Caulobacter elaborate an S-layer visibly indistinguishable from the one produced by Caulobacter crescentus strains CB2 and CB15. The S-lay r prot ins from these strains has approximately 100,000 m.w. The protein has been characterized both structurally and chemically. It is composed of ring-like structures spaced at 22nm intervals arranged in a hexagonal manner on the outer membrane. The S-layer is bound to the bacterial surface by calcium ions and may be removed by low pH treatment or by treatment with a calcium chelator such as EGTA.

The S-layer proteins of S-layer producing strains of Caulobacter have significant similiarity. Thus a cloned S-layer protein gene of one Caulobacter strain will likely be useful to retrieve the corresponding genes in other Caulobacter strains (see: Walker, S.G., S.H. Smith, and J. Smit (1992) "Isolation and Comparison of the Paracrystalline Surface Layer Proteins of Freshwater Caulobacters". J. Bacteriol. 174: 1783-1792; and, MacRae, J.O. and, J. Smit (1991) "Characterization of Caulobacters Isolated from Wastewater Treatment Systems" Applied and Environmental Microbiology 57:751-758).

Expression and presentation of a heterologous polypeptide as a fusion product with an S-layer protein of a bacterium provides several advantages not previously seen in systems using organisms such as E. coli and Salmonella where fusion products of other kinds of surface proteins have been expressed. Firstly, many bacteria producing S-layer proteins (particulary Caulobacter) are relatively harmless and ubiquitous in the environment. In contrast, many Salmonella and E.coli strains are pathogens. Consequently, expression and presentation of a heterologous polypeptide using Caulobacter as a vehicle will have the advantage that the expression system will be stable in a variety of outdoor environments and may not present problems associated with the use of a pathogenic organism. Second, many such bacteria, including Caulobacter, are

natural biofilm forming species and may be adapted for use in fixed biofilm bioractors. Finally, the quantity of the S-layer protein that is synthesized by the bacterium and the unique characteristics of the repetitive, two-dimensional S-layer make such bacteria ideal for use as an expression system and a "presentation surface" for heterologous polypeptides. Such a presentation surface is desirable in a live vaccine so that presentation of a foreign epitope is maximized. In addition, use of the presentation surface to achieve maximal exposure of a desired polypeptide to the environment results in such bacteria being particularly suited for use in bioreactors or as carriers for the polypeptide in aqueous or terrestrial outdoor environments.

Accordingly this invention provides a bacterium having an S-layer wherein the bacterium's S-layer protein gene contains one or more in-frame sequences encoding one or more functional heterologous polypeptides and, said S-layer is a fusion product of the S-layer protein and the said heterologous polypeptide.

This invention also provides the means for producing a bioreactor comprising a suitable substrate (e.g. a rotating biological contactor) with the above described bacterium of this invention adhered thereon. Accordingly, this invention provides such a bioreactor.

This invention also provides a method of expressing and presenting a functional polypeptide which comprises cloning a gene sequence for said polypeptide in-frame into the S-layer protein gene of Caulobacter, and, culturing said Caulobacter having a S-layer which is a fusion product of the S-layer protein and the polypeptide.

This invention also provides a fusion product comprising a S-layer protein and one or more functional

heterologous polypeptide sequences express d according to the prec eding method wh rein, the fusion product is separat d from the Caulobacter cell surface after th expression thereof.

This invention also provides a DNA sequence comprising all or substantially all of a Caulobacter S-layer protein gene containing one or more heterologous sequences encoding restriction endonuclease sites, which heterologous sequences preserve the translational frame of said gene to facilitate insertion of further heterologous sequences into said gene. Plasmids and bacterial cells comprising such a DNA sequence are also provided.

This invention also provides a substantially purified DNA sequence consisting of essentially all or substantially all of a Caulobacter crescentus S-protein gene sequence.

For better understanding of this invention, reference may be made to the preferred embodiments and examples described below, and the accompanying drawings in which:

Figure 1 is the sequence of a carrier cassette which may be cloned into the Pst1/Bam H1 site of pUC9 to deliver a gene sequence of interest to sites within an S-layer protein gene, such as in Caulobacter crescentus.

Figure 2 is a restriction map of a plasmid based promoter-less version of the rsaA gene (pTZ18U:rsaA$_{\Delta}$P) containing restriction sites and which may be used to accept the heterologous DNA of interest.

Figure 3 is the nucleotide sequence of linker BamH1 - 7165K carried in plasmid pUC9B (pUC7165K), which may be used for mutagenesis at sites created in rsaA by a specific or non-specific endonuclease.

Figure 4 is the nucleotide s quence of linker Bam H1 - 6571K carried in plasmid pTZ19 (pTZ6571K) which may b used for mutagen sis at sites created in rsaA by a specific or non-specific endonuclease.

Figure 5 is a map of 15 insertion events at Taq I sites in the rsaA gene identified by the amino acid number of the insertion site in the S-layer protein and scored according to whether the S-layer is produced in the modified organism.

Figure 6 is the complete nucleotide sequence of the C. crescentus rsaA gene and the predicted translational product in the single letter amino acid code. The -35 and -10 sites of the promoter region as well as the start of transcription and the Shine-Dalgarno sequence are indicated. Partial amino acid sequences determined by Edman degradation of rsaA protein and of sequenced peptides obtained after cleavage with V8 protease are indicated by contiguous underlining. The putative transcription terminator palindrome is indicated with arrowed lines. The region encoding the glycine-aspartate repeats is indicated by underlined amino acid code letters. This region includes five aspartic acids that may be involved in the binding of calcium ions. The GenBank accession number is M84760.

A preferred organism for use in this invention is Caulobacter, particularly C. crescentus. Most preferred are C. crescentus strains such as CB15A containing the rsaA gene encoding the paracrystalline S-layer protein as described in: Gilchrist, A. J. A. Fisher and, J. Smit (1992) "Nucleotide Sequence Analysis Of The Gene Encoding the Caulobacter crescentus Paracrystalline Surface Layer Protein". Can. J. Microbiol. 38:193-208.

A heterologous polypeptide r ferr d to herein may be a peptide, polyp ptide, protein r a part of a protein. The heterologous polypeptide may be an enzyme, a ligand, an antigen or another functional sequence of amino acids.

Once a particular bacterium's S-layer protein gene is characterized, this invention may be practiced by implementing one or more known methods of cloning of a desired heterologous gene sequence into the S-layer protein gene so that both the S-layer protein and the heterologous protein are transcribed "in-frame". Knowledge of the S-layer protein gene sequence permits one to identify potential sites to install the heterologous genetic material. The repetitive nature of S-layer protein results in multiple copies of the heterologous protein being expressed and presented on the surface of the cell.

The following general procedure lays out courses of action and specifies particular plasmid vectors or constructions that may be used to accomplish a fusion of an S-Layer protein of interest; and, the positioning of the protein of interest at numerous sites, allowing the determination of the one most suitable for the desired application. The following description uses the rsa A (S-layer) gene of C. crescentus as an example (see Figure 6). The latter gene sequence is characterized in Gilchrist, A.; J.A. Fisher; and, J. Smit (1992) "Nucleotide Sequence Analysis of the Gene Encoding the Caulobacter crescentus Paracrystalline Surface Layer Protein". Can. J. Microbiol 38: 193-202; in contrast to an earlier partial characterization now shown to contain some errors (Fisher, J.A.; J. Smit et al, N. Agabian (1988) J. Bacteriol 170:4706-4713).

The general procedure provides detailed steps allowing for the following possibilities:

A) Use of an available collection of pot ntially permissive sites in th S-layer gen to install the genetic information for th protein of int rest,

B) Use of an available carrier cassette for deliv ring th gen of interest to sites within the S-layer gene. The cassette offers several advantages over the more direct modification of the gene of interest, in preparation for insertion.

C) Creation of a collection of random insertion sites based on a restriction enzyme of choice, if the available collection of potentially permissive sites is for some reason unsuitable.

D) Preparation of the DNA specifying the polypeptide of interest for direct insertion into permissive sites (ie, not using the carrier cassette) by a method best suited for the particular case (several options are suggested).

The general procedure involves the following steps and alternative courses of action.

1) Choose an appropriate region (or specific amino acid position) of the S-layer for insertion of a desired polypeptide.

2) Create a unique restriction site in the (preferably hexameric) rsaA (S-layer) gene at position within the gene encoding that region (or corresponding to a specific amino acid) using either standard linker mutagenesis (regional) or site directed mutagenesis (specific amino acid). The unique restriction site is to later act as a site for accepting DNA encoding the polypeptide of interest. The particular plasmid-based promoter-less version of the rsaA gene (pTZ18U:rsaAΔP) shown in Figure 2 is preferably used because it contains an appropriate combination of 5′ and 3′ restriction sites useful for subsequent steps. The restriction site chosen should not occur in rsaA, its carrier plasmid or, the DNA sequence coding for the polypeptide of interest.

3) If it is unclear which region of th S-lay r would b suitable for insertion of the p ptide/polyp ptide of interest, a random linker mutagen sis approach is used to randomly insert a unique linker-encoded restriction site (preferably hexameric) at various positions in the rsaA gene. Sites for insertion of the linker are created using an endonuclease, either of a sequence specific nature (eg. tetrameric recognition site restriction enzyme) or sequence non-specific nature (eg. Deoxyribonuclease I [DNase I]). A particularly suitable method is the generalized selectable linker mutagenesis approach based on any desired restriction site of: Bingle, W.H., and J. Smit. (1991) "Linker Mutagenesis Using a Selectable Marker: A Method for Tagging Specific Purpose Linkers With an Antibiotic-Resistance Gene". Biotechniques 10: 150-152. Because endonuclease digestion is carried out under partial digestion conditions, a library of linker insertions at different positions in rsaA is created.

If restriction endonucleases are used to create sites for subsequent insertion of the linker encoding a hexameric restriction site, mutagenesis is preferably done with a mixture of 3 different linkers incorporating appropriate spacer nucleotides in order to satisfy reading frame considerations at any particular restriction site (only 1 of the 3 linker insertions will be useful for subsequent acceptance of DNA encoding the polypeptide of interest). With DNase I, only one linker is needed, but again only 1 or 3 linker insertions will be useful for accepting DNA encoding the polypeptide of interest depending on the position of the DNase I cleavage with respect to the 3 bases of each amino acid codon.

Providing Bam HI sites are appropriate as sites for the introduction of DNA encoding the peptide/polypeptide of interest, Bam HI linkers tagged with a kanamycin-resistance gene for selectable linker mutagenesis may be used. One

such 12-bp linker carried in plasmid pUC1021K was describ d by Bingle and Smit (1991) [supra]. Two additional 15-bp linkers (pUC7165K and pTZ6571K) constructed for cr ating the other 2 possible translati n frames within the linker insert itself are described in Figures 3 and 4. Any one of the above three kanamycin-resistance tagged BamHI linkers is suitable for mutagenesis at sites created in rsaA by DNaseI. As outlined above, a mixture of all three linkers is preferably used for mutagenesis at sites created in rsaA by restriction enzyme digestion.

4) Once a library composed of linker insertions (encoding the desired hexameric restriction site) at different positions in rsaA has been created, the DNA encoding the polypeptide of interest is inserted into the sites en masse (the library of mutated rsaA genes may be manipulated as one unit). The library is digested with the restriction enzyme specific for the newly-introduced linker encoded restriction site and ligated to a DNA fragment encoding the polypeptide of interest and carrying the appropriate complementary cohesive termini. The DNA specifying the polypeptide of interest can be prepared by a number of standard methods, which may include oligonucleotide synthesis of 2 anti-complementary strands, polymerase chain reaction procedures, or addition of linkers (whose termini are compatible with the newly-introduced sites in rsaA) to a suitably modified segment of DNA.

In order to facilitate the rapid recovery of useful rsaA genes carrying newly inserted DNA at BamHI sites encoding the polypeptide of interest, the carrier oligonucleotide shown in Figure 1 may be used. The DNA of interest is first directionally cloned, if possible, using the XhoI, Stu 1, or Sal 1 sites or non-directionally cloned using any one of the sites in the same orientation as a chloramphenicol resistance (CmR) gene lacking a promoter. To do this the DNA of interest must be provided with the

appropriate termini for cloning and spac r nucleotid s for maintaining correct reading frame within the cassett and should not contain a BglII sit . For insertion into th BamHI linker library, the DNA of inter st is recovered as a BamHI fragment tagged with a CmR gene. When ligated to the BamHI digested rsaA linker library, only those colonies of the bacterium (eg. E. coli) used for the gene modification steps that are recovered will be those carrying insertions of the desired DNA in the correct orientation, since the promoter on the plasmid is 5′ to rsaAΔP and the CmR gene. This eliminates screening for DNA introduction and increases the recovery of useful clones by 100% (1 of 3 versus 1 of 6). While still manipulating the library as one unit, the CmR gene is removed using BglII. The carrier oligonucleotide also provides the opportunity to add DNA 5′ or 3′ to the DNA of interest at Sal I, XhoI or Stu I sites providing the DNA of interest does not contain any of these sites. This allows some control over spacing between rsaA sequences and and the sequence of the DNA of interest.

5. The rsaA genes carrying the insertion of the DNA of interest in the correct orientation is excised from the plasmid (eg. from the pTZ18U:rsaAΔP plasmid) and is transferred to a suitable vector providing a promoter recognized by Caulobacter. Preferably, such a vector is pWB9 or pWB10 (Bingle, W.H., and J. Smit (1990) "High Level Plasmid Expression Vectors for Caulobacter crescentus Incorporating the Transcription and Transcription-Translation Initiation Regions of the Paracrystalline Surface Layer Protein Gene". Plasmid 24: 143-148) with EcoRI/SstI sites. Therefore, the DNA of interest should not contain the latter sites. These vectors allow expression of rsaA hybrids in S-layer negative mutants of C.crescentus such as CB15KSac (Edwards, P. and, J. Smit (1991) "A Transducing Bacteriophage for Caulobacter cresenctus Uses The Paracrystalline Surface Layer Protein

As a Receptor" J. Bacteriol. 173:5568 -5572); or, CB2A described in: Smith, J. and, N. Agabian (1984) J. Bacteriol. 160:1137 -1145.

Those Caulobacters surviving transfer are examined for S-layer assembly and presentation of the new polypeptide activity, antigenicity, etc. by methods specific to the needs of the investigator or the capabilities of the inserted sequence. Many of the sites created are "benign" as they have no effect on the functional regions of the protein involved with export, self assembly, etc. However, not every site that results in an absence of functional disruption of the S-layer is best for insertion of new activities. Some sites may not be well exposed on the surface of the organism and other sites may not tolerate insertion of much more DNA than the linker sequence.

Use of the S-layer protein as a vehicle for expression and presentation of a heterologous polypeptide has several advantages. Firstly, the S-layer protein is synthesized in large quantities and has a generally repetitive sequence. This permits the development of systems for synthesis of a relatively large amount of heterologous material as a fusion product with the S-layer protein. It may be desirable to retain the fusion product as part of the bacterial cell envelope or, the fusion product may be separated from the organism, such as by the method described in: Walker, S.G.; S.H. Smith; and J. Smit (1992) "Isolation and Comparison of the Paracrystalline Surface Layer Proteins of Freshwater Caulobacters". J. Bacteriol. 174: 1783-1792. Alternatively, the Caulobacter strain that is used to express the fusion product may be derived from a strain such as CB15Ca5 that sheds its S-layer (Edwards and J. Smit (1991) [Supra]).

Second, this invention is particularly suitable for use in bioreactor systems. An example is the use of a

modified Caulobacter in a bioreactor to bind toxic metals in sewag , wast wat r tc. Caulobacters are ideal candidates for fixed-cell bioreactors, th construction of which is well known. An example of such a bioreactor is a rotating biological contactor. Although other bacteria are found in the environment that are capable of binding metals, they often do so by producing copious polysaccharide slimes that quickly plug filtration systems. In some cases, the bacteria are not surface-adherent or the bacteria does not show selectivity towards key toxic metals.

Metal metallothioneins are small cysteine-rich proteins induced by many organisms in response to exposure to heavy metals. They are generally expressed internally and are designed to limit exposure of other aspects of cell physiology to the toxic metals. Typically, metallothioneins are composed of about 60 amino acids and the genes from a variety of mammalian organisms have been cloned and sequenced. Metallothioneins bind metals such as cadmium, zinc, cobalt, copper and mercury in significant amounts (such as from 4-12 moles of metal per mole of protein). Modification of a Caulobacter such that its S-layer protein is a fusion product with a metallothionein would provide a suitable component for a bioreactor. By taking advantage of the natural bio-film forming characteristics of Caulobacter, bioreactors may be formed comprising a substrate and a single layer of cells adhered thereon, with the cells distributed at high density. A variety of substrates may be used such as a column of chemically derivatized glass beads or a porous ceramic material (such as ceramic foam).

Another advantageous application for this invention is in the production of batch cultures of modified Caulobacter wherein the S-layer protein is a fusion product with an enzyme, such as xylanase or cellulase. Such Caulobacter

could be grown in wood pulp suspensions at an appropriate juncture of the pulping process in order to provide for partial decomposition of the wood-pulp structure. Such an application may permit more effective penetration of bleaching agents in the wood-pulp bleaching process thereby reducing the use of chlorine-based bleaching agents.

Another advantageous application of this invention is the production of organisms that present vaccine-candidate proteins at the organism's cell surface. For example, modified Caulobacter that are relatively safe to handle and which are readily cultured in outdoor freshwater environments, would be particularly useful for fish vaccines. The two-dimensional crystalline array of the S-protein layer of Caulobacter, which has a geometrically regular, repetitive structure, provides an ideal means for dense packing and presentation of a foreign epitope to an immune system.

Example 1: Production of Permissive Insertion Sites in C.cresentus

Using the restriction enzyme TaqI, a partial digestion of the rsaA gene in pTZ18U:rsaAΔP produced a group of linearized segments with random TaqI sites cleaved. The linearized segments were modified by use of the tagged linker mutagenesis procedure of Bingle and Smit (1991) [supra], using the 12-bp Bam HI linker carried in plasmid pUC102K discussed in the general procedure above. Those products that produced a full-length protein in E. coli were ultimately transferred to pWB 1, (a minor variation of pWB9), a vector that is replicated by Caulobacter, again as described in the general procedure. The resulting construction was introduced into a C. crescentus strain. Fifteen distinguishable events were retrieved and analyzed for the ability to produce a full-length protein in C.crescentus and to produce the crystalline S-layer on their surface. The results of this screening and the

position of nine successful events ar illustrated in Figure 5.

The above-described nine positive events represent cases where the 4-amino acid insertion is tolerated with no effect on the S-layer function. Thus, they have a higher potential for tolerating the addition of more foreign peptide material than less characterized sites. By producing 3 versions of the gene of interest, representing each possible reading frame (using standard linker addition technology), one may test each of these sites for suitability in expressing the desired activity. By using restriction enzymes other than Taq I, such as Aci I, Hin PI or Msp I, a larger library of Bam HI insertions may be created.

In the case of each or the above described nine positive events, S-layers were produced on the Caulobacter that were indistinguishable from wild-type layers.

Example 2: Insertion of Metallothionein Into a Specific Site

An insertion of the above described 12bp linker was made at the Taq I site that corresponds to amino acid #188, frame #3 (see Figure 6). This created a unique Bam H1 site at that position. Because the precise position of the Taq I site could be assessed from the DNA sequence information available for the rsaA gene, the necessary translation frame was known and thus a single construction of the metallothionein gene was made. This was done by excision of the coding sequence of monkey metallothionein II peptide (which is 60 amino acids with a molecular weight of about 5000) at known restriction sites and adapting the gene ends with Bam H1 linkers with appropriate base pair spacers for the needed translation frame.

After insertion into the Bam H1 sit created at position 188, frame 3, sev ral clones w re examin d by d termining whether they could bind levated levels of cadmium. This is a functional assay for the metallothionein, explained in more detail below. The assay was necessary because the segment had equal probability of being inserted backwards. One clone that gave positive results was examined by electron microscopy and the presence of a normal S-layer was confirmed. The plasmid in the clone that gave positive results was also examined by DNA sequencing analysis, sequencing across the junction between the position 188 site and the 5′ side of the metallothionein gene. The sequence data confirmed correct orientation.

The plasmid-containing clone and relevant control strains were examined for the ability to bind several metals known to be bound by native metallothionein. This was done by growing the strains of bacteria in the presence of the metals at a concentration of 5ug/ml. After extensive washing of the cells to remove unbound metal, the cells were ashed by treatment at 500°C and the residue was dissolved in dilute nitric acid and examined for metal content by atomic absorption spectrosocopy. The results from one round of data collection is shown in Table 1. In the case of cadmium and copper, an elevated level of bound metal is noted in the metallothionein-expressing strains.

Table 1

Caulobacter	Metal Ion Tested (ug/metal/OD unit of cells			
	Copper		Cadmium	Zinc
	Trial 1	2		
CB15 (wild-type,S-layer(+))	1.79	1.0	0.71	4.15
CB15XSac (S-layer negative strain)	2.18	1.33	1.07	4.08
CB15XSac/p188.3 (contains S-layer with linker insert only)	2.02	1.30	11.1	3.66
CB15XSac/p188.3MT (S-layer with Metallothionein inserted)	2.79	3.09	19.1	3.00

This invention now being described, it will be apparent to one of ordinary skill in the art that changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

Figure 1 The Carrier Cassette

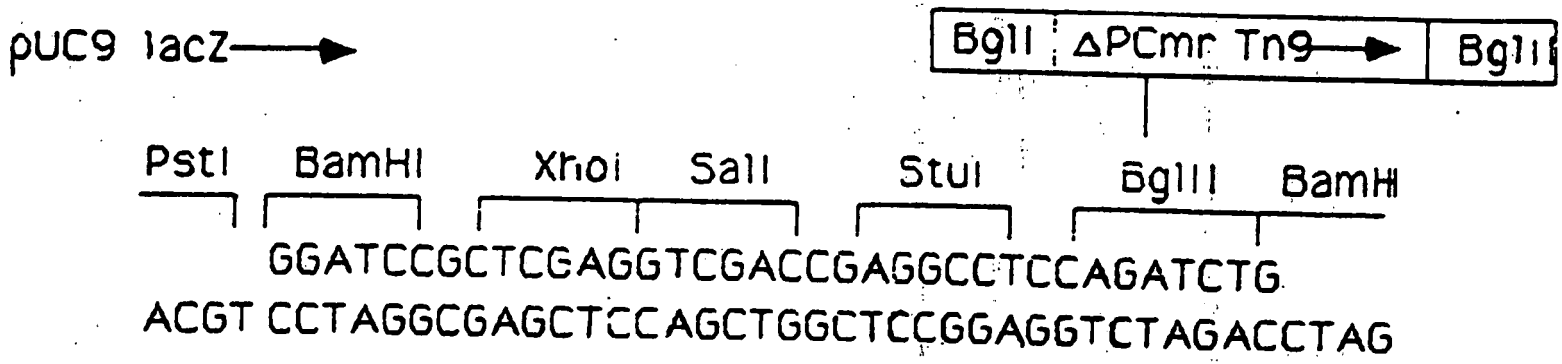


Figure 2: Restriction Map of pTZ18U:rsaAΔP

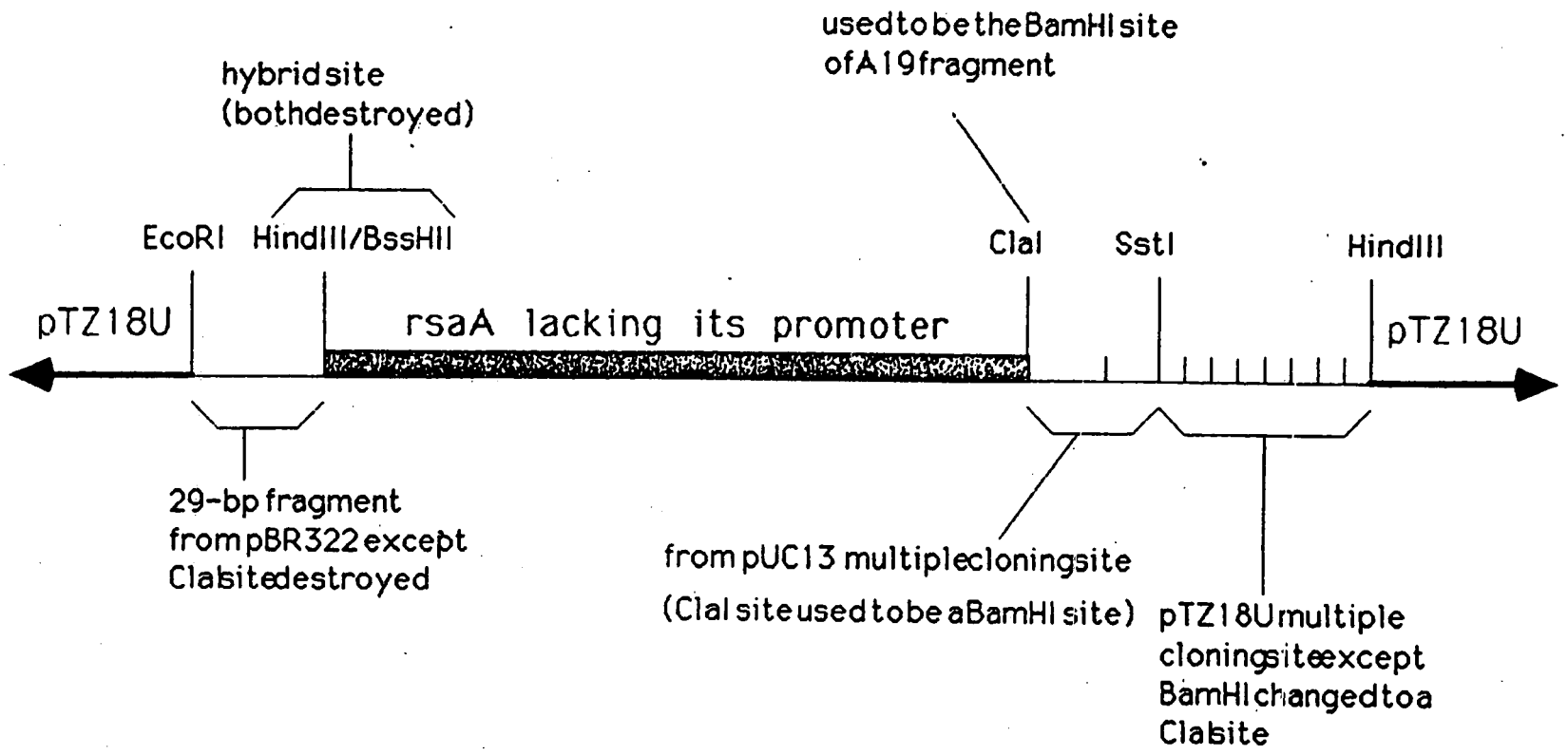
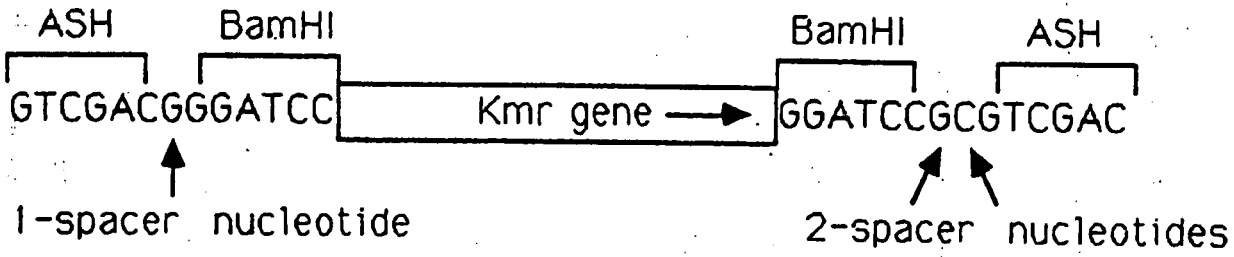
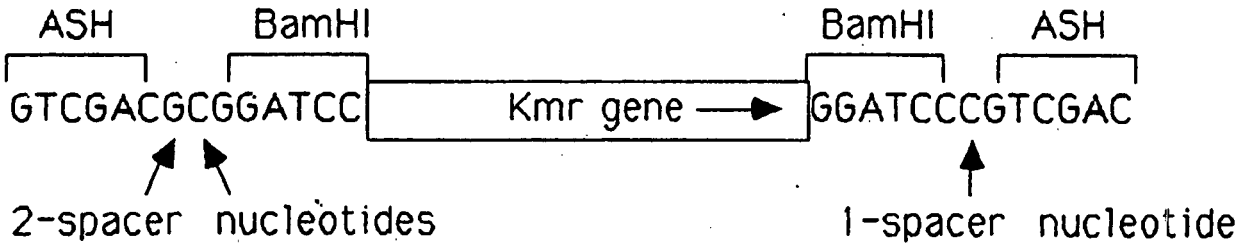


Figure 3: Deduced nucleotide sequence of linker BamHI-7165K carried in plasmid pUC9B (pUC7165K)



ASH= AccI/SalI/HincII restriction site

Figure 4.: Deduced nucleotide sequence of linker BamHI-6571K carried in plasmid pTZ19 (pTZ6571K)



ASH=AccI/SalI/HincII site

FIGURE 5. Scoring of linker-insertion events at Taq I sites in the rsaA gene

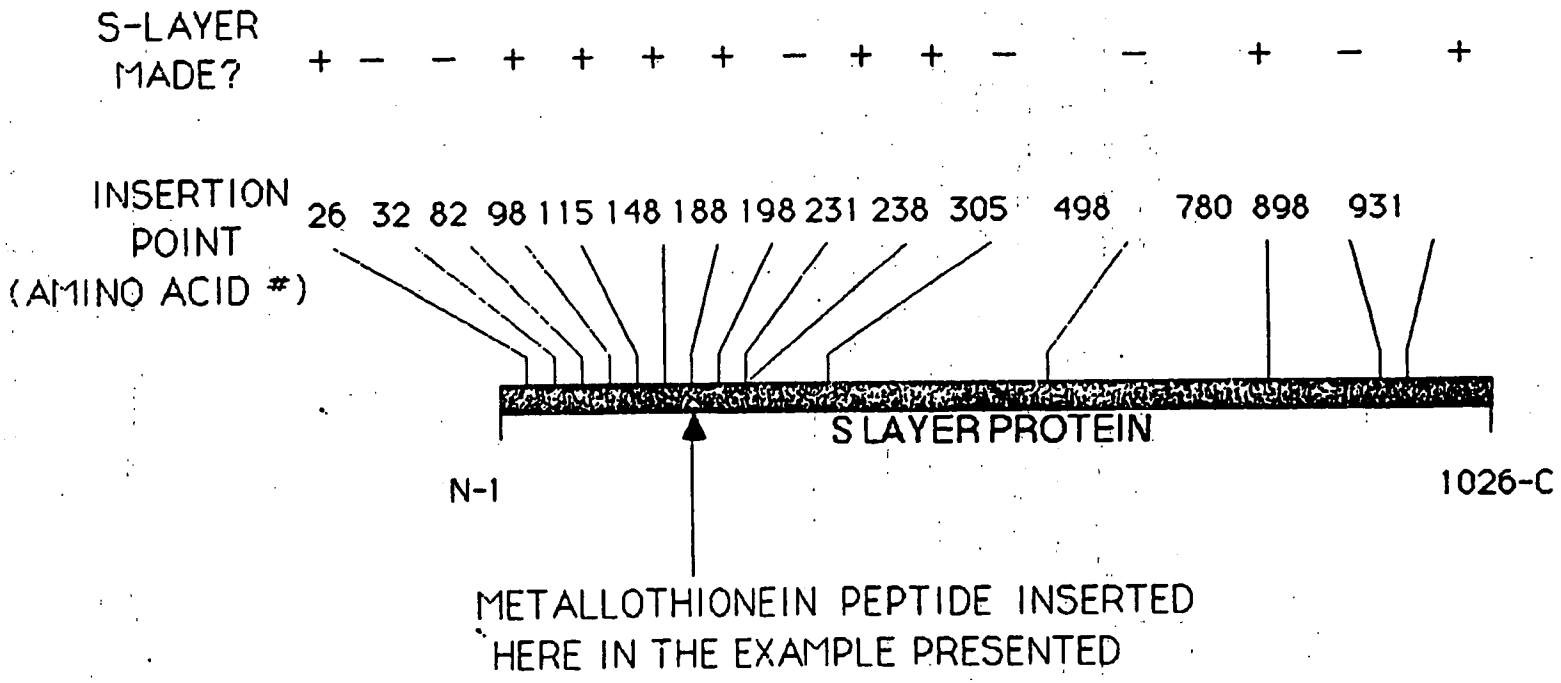


FIGURE 6

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